THE IDENTIFICATION AND RANKING OF POLY (ADP-RIBOSE) POLYMERASE INHIBITORS AS PROTECTORS AGAINST SULFUR MUSTARD INDUCED DECREASE IN CELLULAR ENERGY AND VIABILITY IN IN VITRO ASSAYS WITH HUMAN LYMPHOCYTES

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ABSTRACT

Lymphocyte were utilized as a model for investigating HD effects on resting cells. Lymphocytes exposed to HD demonstrated a concentration dependent decrease in ATP, NAD, and viability. The decrease began in 15 minutes for ATP, 2 hours for NAD, and 6 hours for viability. All three of these HD initiated biochemical changes can be blocked by poly (ADP-ribose) polymerase inhibitors (PADPRPI). To completely inhibit HD initiated ATP, NAD, and viability decreases the PADPRPI had to be present at time 0, 1, and 4 hours respectfully. The amount of protection conferred by the PADPRPI in the viability assay decreased in a linear manner with the delay of the addition and the concentration of the inhibitor from 6-12 hours post HD exposure. There was a good correlation between IC_{50} to inhibit poly (ADP-ribose) polymerase and EC_{50} prevention of HD initiated cell death (r=0.94). Thus, three in vitro assays which can measure biochemical and pathologic changes induced by HD in G lymphocytes have been developed. These assays have been employed to study the ability of candidate antidotes to prevent HD initiated changes. Benzamidine analogs, including the F.D.A. approved vitamin niacinamide, have been shown to be effective at inhibiting all of these changes.

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INTRODUCTION

Sulfur mustard (2,2'-dichlorodiethyl sulfide, HD) is a potent vesicant which can cause severe lesions to skin, lung, and eyes. Due to the high number of debilitating exposures to HD during the Iran-Iraq war there is an increased interest in its mechanism of action and in the development of therapeutic intervention(s). A Decision Tree Network (DTN) has been proposed and is currently under development for determining the efficacy of candidate antidotes to prevent HD-induced damage. The DTN is a three phase system: Phase I - in vitro cell culture assays; Phase II - In vitro organ culture assays; and Phase III - In vivo based systems utilizing two animal models.

Phase I is designed to measure the ability of a wide variety of potential antidotes to prevent HD-induced injury including the following: scavengers, poly (ADP-ribose) polymerase inhibitors, NAD level stabilizers, cellular energy suppliers, secretory inhibitors, inflammatory mediator inhibitors, protease inhibitors, DNA repair stimulators, cell cycle regulators and membrane stabilizers. The initial three assays in Phase I, compound cytotoxicity, cellular viability after HD exposure, and changes in biochemical marker (i.e., ATP) assays have been developed and validated. The cytotoxicity assay is used to determine the highest concentration of a candidate antidote to cause less than a 10% decrease in the viability of human lymphocyte. The cell viability assay is used to determine the concentration of a candidate antidote which will protect 50% of the cells from death due to HD-exposure. The biochemical marker assay is used to determine the concentrations of candidate antidote which will prevent 50% of the HD-induced biochemical change (i.e., decreases in ATP, NAD levels) from occurring.

To develop the viability and biological marker assays, the change in the parameter of interest had to be correlated to concentration and time dependent effects of the HD exposure.

METHODS

Materials. The following reagents and chemicals were purchased: Reagents for making routine buffers (Fisher Scientific Co., Pittsburgh, PA); RPMI 1640 medium modified with L-glutamine and 25mM HEPES (RPMI 1640M); garamycin (Whittaker M.A. Bioproducts, Walkersville, MD); Percoll (Pharmacia Inc., Piscataway, NJ); trypan blue, propidium iodide, niacinamide, niacin, 3-aminobenzamide, ATP monitoring reagent, ATP (Sigma Chemical Co., St. Louis, MO). A Becton Dickinson FacStar Plus Flow Cytometer and a LKB Luminometer

1200 were used to determine viability and chemiluminescence, respectively.

HD was obtained through the Chemical and Biological Defense Agency, Aberdeen Proving Grounds, MD, and was assayed by gas chromatography as >99% pure.

The poly (ADP-ribose) polymerase inhibitors evaluated in these studies were obtained from medicinal chemistry contracts monitored by the Division of Experimental Therapeutics, Walter Reed Army Institute of Research, from government archives of previously synthesized compounds, and from gifts from industry.

Isolation of human lymphocytes. Up to 200 ml of blood was drawn from normal human volunteers. The lymphocytes were isolated from blood by Percoll density centrifugation (density=1.080 at 20°C) (MacGlashan and Lichtenstein, 1980). The distinct mononuclear cell layer created by centrifugation, was collected, washed and diluted to a final concentration of $2x10^7$ cells/ml.

Exposure of human lymphocytes to HD. Lymphocytes were allocated into 1.5ml Eppendorf test tubes or 96 well plates ($1x10^6$ cells/tube or well). HD was freshly prepared in ice cold RPMI 1640M to decrease hydrolysis and added directly to the test tubes or plate so that the final volume was 200µl. The test tubes were placed in a 37°C water bath or incubator in a hood for varying times.

Determination of the effectiveness of the various inhibitors at reducing HD-induced loss in cell viability. The inhibitors were screened in the cell viability assay to determine the lowest concentration that prevented cell death at 24 hr after exposure. Triplicate cell samples were pretreated with five non-cytotoxic concentrations of inhibitor (at decreasing 1/2 log intervals) and exposed to 170 µM HD. The challenge concentration of 170 µM HD (EC87) was chosen so that the screening assay would be sensitive to small effects of the inhibitor. Cell viability was determined at 24-26 hr post exposure to HD.

Flow cytometry analysis of cell viability. At 24-26 hr post-exposure, 50 µl of propidium iodide (20 ug/ml in RPMI 1640 media) added to the wells and allowed to incubate for 3 min at room temperature. The plates were then placed in a FACSMate attachment (Becton Dickinson, San Jose, CA) to a FACStar Flow cytometer (Becton Dickinson, San Jose, CA) for viability analysis. The FACSMate permits automatic sampling of all wells of the 96-well plate. Data were collected from 10,000 cells in

each sample. The flow cytometer operates with a 5 W argon laser generating a 488 nm line at 200 mW.

Determination of ATP levels. At the end of the incubation, 10µl of a 20% trichloroacetic acid solution was added to the tubes. The mixture was incubated for 15 minutes at room temperature and neutralized. 25µl of lymphocyte extract was added to a cuvette and the ATP measured by the LKB procedure (Thore, 1979).

Data Analysis. Flow cytometry data from each sample were analyzed using the Lysis II software program (Becton Dickinson) to determine percent cell viability. Mean percent viability values and standard deviations were determined from 3 exposures per experimental run. Percent response values were calculated as follows:

$$\frac{X - X_{max}}{X_c - X_{max}} \times 100$$

where:

X = mean % viability of sample group

X_{max} = mean % viability of maximum response group

X_c = mean % viability of the non exposed vehicle control group.

RESULTS

Effect of HD concentration on lymphocyte viability. To select a challenge concentration of HD to test inhibitor effectiveness, a well defined concentration-response relationship for HD-induced loss in cell viability was determined. The data from 4 experimental runs were pooled (Fig. 1) and examined using the probit method to calculate the EC_{50} and determined the linear portion of the curve (Finney, 1971). Human lymphocyte viability was unaffected following exposure to 10 µM HD or less. A linear decrease in cell viability occurred between 13 µM (EC_{16}) and 173 µM (EC_{97}) HD, and plateaued at HD concentrations of 200 µM and above. The EC_{50} was calculated to be 44 µM HD. The percentage of cells dying at this plateau was variable among the lymphocyte preparations examined, but did not drop below 35 percent of control viability, suggesting that a fraction of cells in these preparations were resistant to the effects of HD.

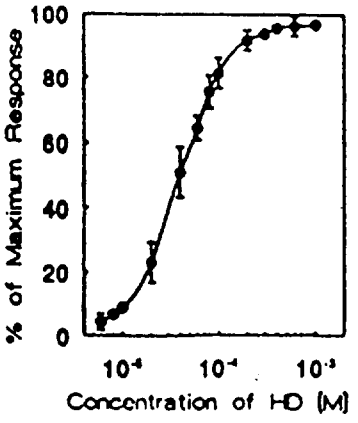

Figure 1. Effect of HD concentration on lymphocyte viability was determined at 24 hr post exposure to the indicated concentrations of HD. Data from 4 experiments are pooled and plotted. Error bars represent SEM of the 4 experiments.

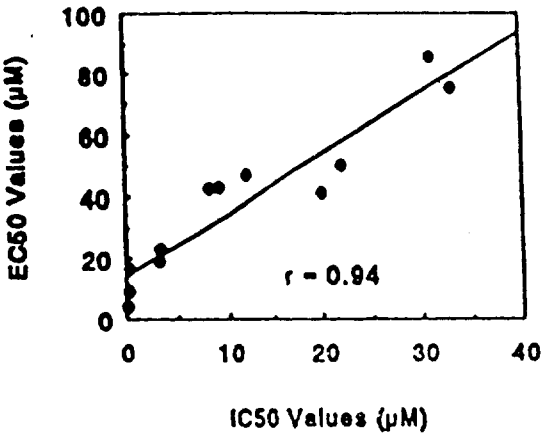

Figure 2. Correlation of the ability to reduce HD-induced cell death with the ability to inhibit PADPRP activity. EC_{50} values for the reduction of HD-induced loss in cell viability were plotted verses reported IC_{50} values for inhibition of PADPRP. The data were examined by linear regression analysis and a correlation coefficient (r) was calculated.

Effectiveness of the various inhibitors at reducing HD-induced loss in cell viability. To determine whether inhibitors of ADP-ribosylation are effective at reducing HD-induced cell death, 12 inhibitors of PADPRP and 5 inhibitors of MADPRT (Mono ADP-ribose transferase) were examined. The PADPRP inhibitors were selected based on reports that they were more potent inhibitors of PADPRP than niacinamide and 3-aminobenzamide, and that they were more selective for PADPRP than for MADPRT (Rankin et al., 1989; Suto et al., 1991; Banasik et al., 1992; Leopold et al., 1992). All of the inhibitors of PADPRP were found to be effective at reducing HD-induced cell death and were found to be more potent than niacinamide and 3-aminobenzamide. There was a significant correlation ($r = 0.94$) between the ability to inhibit PADPRP (based on reported IC50 values) and the ability to reduce HD-induced cell death (Fig. 2) for the 12 PADPRPI tested; however, there was no correlation between MADPRT inhibition and reduction in HD-induced cell death (data not shown). To determine if inhibitors of lipid peroxidation were effective at reducing HD-induced loss in cell viability, allopurinol, U75412E, U74500A, and U78518E were examined at concentrations between 1 μM and 100 μM. Although these compounds are reported to be potent inhibitors of lipid peroxidation, none of these compounds were effective at reducing HD-induced loss in cell viability (data not shown). In addition, cycloheximide, a potent inhibitor of protein synthesis, was examined at concentrations between 1 μM and 1000 μM. Cycloheximide was also ineffective at reducing HD-induced cell death (data not shown).

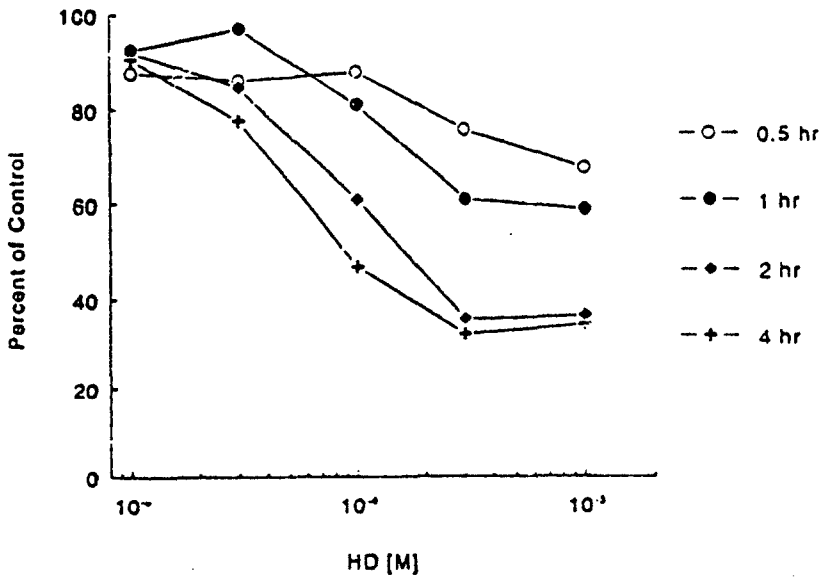

Figure 3. The time-and concentration-dependence of HD on the level of ATP in exposed human lymphocyte preparations was studied. Concentrations of HD between 10^{-5} and $3x10^{-4}$M demonstrated a concentration-dependent decrease in ATP from 1 to 4 hr post-exposure. All points are the mear of 3 separate tubes. This experiment represents 3 experiments with similar results.

Effects of HD exposure on ATP levels of human lymphocyte preparations. Human lymphocytes were exposed to HD at concentrations of 10^{-5} to $3x10^{-4}$M and ATP levels were measured after 0.5, 1, 2, 4, 6, 8, and 20 hours. ATP levels of the HD exposed cells dropped over this concentration range to about 20% of control levels by 8 hrs and remained at that level over the 20 hrs of the experiment. Although there was some concentration dependent effects observed at the lower concentration of HD 6 hr after HD exposure, it did not cover the entire concentration range (data not shown). However, between 1-4 hours post HD exposure there was a correlation between the concentration of HD and the decrease in lymphocyte ATP levels over the entire range of 10^{-5} to $3x10^{-4}$M HD (Figure 3). The rate of decrease in the ATP levels was proportional to the concentration of HD to which the cells were exposed.

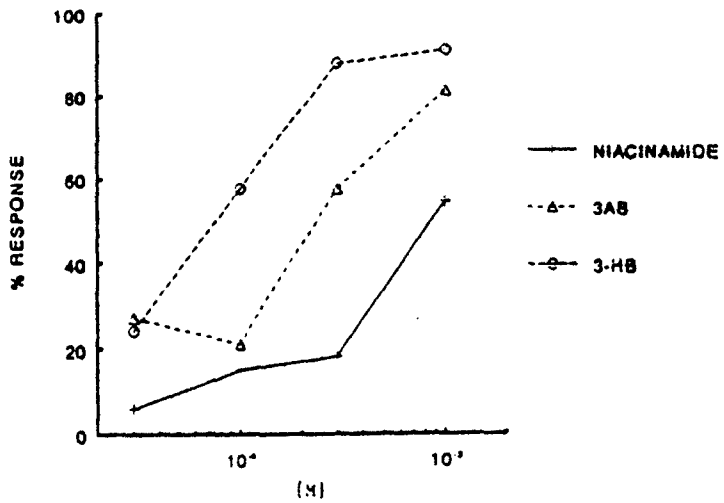

Figure 4. The ability of PADPRPIs (niacinamide, 3-aminobenzamide, and 3-hydroxybenzamide) to protect human lymphocyte ATP levels from HD-initiated depletion was studied. The cells were exposed to the indicated concentration of inhibitor and 10^{-4}M HD. ATP levels were determined at 2 hrs. All points are the mean of 3 separate tubes. This experiment represents 3 experiments with similar results.

The effect of candidate antidotes on the HD-dependent decrease in ATP levels. Based on the concentration- and time-dependent results discussed above, experiments were designed to determine the efficacy of candidate compounds in preventing the fall in ATP 2 hrs after exposure to 10^{-4}M HD. Niacinamide, both an inhibitor of poly (ADP-ribose) polymerase (IC_{50} of 31μM) and a substrate for NAD synthesis, demonstrated partial protection of lymphocyte ATP levels in the 2 hour incubation at concentrations between $3x10^{-5}$ to 10^{-3}M when added at the time of HD exposure (Figure 4). 3-Amino-benzamide, an inhibitor of poly (ADP-ribose) polymerase (IC_{50} of 33μM) but not a substrate for NAD synthesis, paralleled the activity of niacinamide. 3-Hydroxybenzamide, approximately a 3-fold more potent inhibitor of poly (ADP-ribose) polymerase (IC_{50} of 9.1μM) than either niacinamide or 3-aminobenzamide but not a substrate for NAD synthesis, was much more effective at preventing HD induced decrease in cellular ATP levels than either niacinamide or 3-aminobenzamide. Niacin, a substrate for NAD synthesis but not an inhibitor of poly (ADP-ribose) polymerase, conferred no protection against HD-induced decrease in cellular ATP levels at concentration as high as $3x10^{-4}$M.

Allopurinol, an inhibitor of xanthine oxidase (IC_{50} of 0.19μM) (Nukatsuka, 1990) was also tested for its ability to prevent HD-dependent decrease in human lymphocyte ATP levels. It conferred no protection against 10^{-4}M HD induced decrease in cellular ATP levels at concentration as high as $3x10^{-4}$M.

DISCUSSION

Due to the expanded production and utilization of HD in the world (Norman, 1988), there is an increased risk that more individuals will be exposed to HD in the future. To develop therapeutic intervention(s) effective at preventing HD-induced cutaneous injury, it is necessary that the mechanism of action of HD on human cells be better understood. Dr. Papirmeister, while at our laboratory, proposed a hypothesis (Meier et al., 1984; Papirmeister et al., 1985) based on the Berger suicide hypothesis of DNA mediated cell death (Berger et al., 1979). The hypothesis states that HD extensively alkylates DNA in cells, resulting in extensive depurination of the DNA strands (Fox and Scott, 1980; Wheeler, 1962). The resulting DNA breaks cause an increase in the activity of the chromosomal enzyme poly (ADP-ribose) polymerase (Berger et al., 1979). Poly (ADP-ribose) polymerase uses the cofactor NAD^+ as a substrate and polymerizes the ADP-ribose moiety from NAD^+ on different protein receptors (Hayaishi and Ueda, 1982). Hyperactivity of this enzyme can deplete the cells of NAD^+, which leads to an inhibition of glycolysis (Barron, 1948) the main source of energy for the epidermis (Freinkel, 1960). The inhibition of glycolysis should result in a decrease in cellular ATP levels and eventual cell death.

In attempt to study and test this hypothesis, we initially demonstrated in human lymphocytes that HD causes a decrease in cellular NAD levels (Meier et. al., 1987). It was found that the HD-initiated decrease in NAD could be blocked either by PADPRPIs, niacinamide and 3-aminobenzamide, or by a NAD synthesis substrate, niacin, (Meier et. al., 1987). In this report we investigated, the effects of HD on cellular energy (ATP) and cellular viability in human lymphocytes. As predicted by the Berger-Papirmeister hypothesis, we have demonstrated both a time-and concentration- dependent decrease in both ATP and cellular viability. Also in agreement with the hypothesis is that the decrease in both of these parameters of HD exposure could be prevented by PADPRPIs, niacinamide, 3-aminobenzamide, and 3-hydroxybenzamide but not protected by NAD synthesis substrates, niacin, nor inhibitors of lipid peroxidation, allopurinol. The ability of the PADPRPIs to prevenc the decrease in cellular viability due to HD appears to strongly correlate with its potency as an inhibitor of PADPRP.

There also appears to be a correlation between the IC_{50} for PADPRPI and protection of cellular ATP levels though more PADPRPI will have to be tested before that conclusion can be substantiated.

Our results suggest that the time course of the decrease in NAD and ATP is the opposite of what would be expected based on the hypothesis. The hypothesis predicts that the decrease in NAD should precede and cause the decrease in ATP. However, the decrease in ATP begins as early as 15 min. post HD exposure while the decrease in NAD does not occur until 1 hr. post HD exposure (Meier et. al., 1987). Further studies are needed to elucidate the role NAD and ATP depletion has in the mechanism of HD induced injury.

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